GR

ORIGINAL SUBMISSION

GRAS Notification for the Use of Lactic Acid Bacteria to Control Pathogenic Bacteria in Meat and Poultry Products

Volume 1 of 1

Submitted by:

Nutrition Physiology Corporation
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June 6, 2005

Cover Letter

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June 6, 2005

Laura Tarantino, Ph.D., Director
Office of Food Additive Safety, (HFS 200)
Center for Food Safety and Applied Nutrition
5100 Paint Branch Parkway
College Park, MD 20740

VIA: Courier

Re: GRAS Notification for Lactic Acid Bacteria (LAB) Mixture

Dear Dr. Tarantino:

Pursuant to the proposed 21 CFR § 170.36 (c) Nutrition Physiology claims that the use of Bovamine® Meat Cultures is exempt from the premarket approval requirements of the Federal Food, Drug and Cosmetic Act because we have determined by scientific procedures that such use is Generally Recognized as Safe (GRAS) as a processing aid, acting as a competitive inhibitor to pathogenic organisms in meat and poultry products.

In accordance with proposed regulation, the following information is provided:

Proposed 21 CFR § 170.36 (c)(1)(i) The name and address of the notifier:

> Nutrition Physiology Corporation (NPC)
> 11358 Woods Bay Lane
> Indianapolis, IN 46236

Proposed 21 CFR § 170.36 (c)(1)(ii) The common or usual name of the notified substance:

> Lactic Acid Bacteria (LAB) Mixture
> Trade Name: Bovamine® Meat Cultures

Proposed 21 CFR § 170.36 (c)(iii) The applicable conditions of use of the notified substance:

> For control of *E. coli, Salmonella., Listeria* and other pathogenic bacteria in fresh chopped/ground, whole muscle cuts, and carcasses of meat and poultry.

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Proposed 21 CFR § 170.36 (c)(1)(iv) The basis for the GRAS determination:

This GRAS determination is based on scientific procedures.

Proposed 21 CFR § 170.36 (c)(1)(v) Availability of information:

A summary of the data and information supporting this GRAS notification is attached. If you have any questions or require additional information, please contact Dr. Clyde A. Takeguchi, Ph.D. at Phoenix Regulatory Associates, Ltd., 21525 Ridgetop Circle, Suite 240, Sterling, VA 20166 by telephone at (703)-406-0906 or by email at phoenix@phoenixrising.com.

Sincerely,

Douglas R. Ware, Ph. D., President
Nutrition Physiology Corporation

Attachment: Original and two (2) copies

cc: R. Post, FSIS: letter and three (3) copies
Phoenix Regulatory Associates, Ltd. letter only

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GRAS

Notification

GRAS Notification for the Use of Lactic Acid Bacteria to Control Pathogenic Bacteria in Meat and Poultry Products

1. Introduction

Lactic acid bacteria (LAB) species have a long history of safe use in food and food products, in direct fed microorganisms in animal feed, and as probiotics in dietary supplements. LAB are used in starter cultures for sausage making and are recently being used as protective cultures based on the inhibition of undesired or pathogenic microorganisms by competition for nutrients, by the production of antimicrobial metabolites, or by other specific mechanisms.

The concept of competitive exclusion for the proposed uses is to use LAB that do not grow at refrigeration temperatures but give an inhibitory effect due to the continuous production of metabolites by the cells during the storage.

2. Identity

2.1 Common and Usual Name

Lactic Acid Bacteria (LAB) Mixture
Trade Name: Bovamine® Meat Cultures

2.2 Identity of Microorganisms

2.2.1 *Lactobacillus acidophilis* (NP35, NP51)

LAB were isolated from fecal samples from cattle that were culture negative for *E. coli*. Isolate NP35 (M35) was selected as the best candidate for a competitive exclusion product. Isolate M35 was identified as *L. acidophilis* by the Analytical Profile Index evaluation of carbohydrate utilization (API) and as *L. crispatus* by 16S rRNA analysis (Brashears *et al.* 2003).

NP51 (LA-51; 381-IL-28) is a commercially available strain originally isolated from a calf and identified as *L. acidophilis* (Brashears *et al.* in press).

GRAS Notification – Use of Lactic Acid Bacteria (continued)
2. Identity (continued)

2.2.2 *Lactobacillus lactis* (NP7)

LAB were isolated from alfalfa seeds and sprouts. Isolate NP7 (L7) was identified as *L. lactis* subspecies *lactis* by the API system and selected as the best candidate for a competitive exclusion product (Wilderdyke *et al.* 2004). Inhibitory activity was attributed to organic acids and peptides.

2.2.3 *Pediococcus acidilactici* (NP3)

LAB were isolated from ready-to eat meats (ham and frankfurters). Isolate NP3 (D3) was identified as *P. acidilactici* and selected as one of the best candidates for a competitive exclusion product (Amezquita and Brashears 2002). Inhibitory activity was attributed to organic acids and peptides.

3. Manufacturing

Batches of bacteria have been cultured in a pilot plant setting. Commercialization will require scale-up of the culturing process.

3.1 Growing Conditions

Bacteria are cultured in media specifically designed for each organism by using a 1% inoculum at a temperature range between 35° C to 42° C. The base formulation for culturing microorganisms is the NPC-1 media, consisting of tripticase, casamino acids, yeast extract, and safe and suitable media components. Additions are made on a per strain basis. Glucose and lactate are made as additions to the media depending upon organism.

3.2 Production

The culture time for each strain varies but it takes approximately 20 h from inoculation until late stationary phase. The production process is summarized below and in the attached flow diagrams describing the 1) seed strain maintenance, 2) stock culture production, and 3) finished production [See Figures 1-3].

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4. Conditions of Use

4.1 Purpose

For control of *E. coli, Salmonella., Listeria* and other pathogenic bacteria in fresh chopped/ground, whole muscle cuts, and carcasses of meat and poultry.

4.2 Level of Use

10^6 to 10^8 cfu lactobacilli/g

4.3 Population Expected to Consume the Substance

General population

5. Safety and Effectiveness

5.1 History of Use

LAB species have a long history of safe use in food and food products. Currently, prior-sanctioned uses of LAB starter cultures are allowed for breads (21 CFR Part 136 Bakery products), cultured milk products (21 CFR 131.112 Cultured milk, 131.160 Sour cream, and 21 CFR 131.200 Yogurt), cheese (21 CFR 133.128 Cottage cheese and 21 CFR 133.113 Cheddar cheese), and bacon and sausage products (9 CFR 424.21 Use of food ingredients and sources of radiation).

The use of LAB as competitive inhibitors to undesired or pathogenic microorganisms has been known for several decades. FDA allows LAB and other safe and suitable microorganisms as direct-fed microorganisms in animal feed (36.14 Direct-fed microorganisms; Official Publication, AAFCO, 2004) and as probiotics in dietary supplements. In recent years, the use of LAB as "protective cultures" rather than starter cultures have gained importance. Brashears *et al.* (in press) have reviewed their uses in animal feeding to improve food safety. They state that this concept of microbial antagonism or microbial interference is based on the inhibition of undesired or pathogenic microorganisms by competition for nutrients, by the production of antimicrobial metabolites or by other specific mechanisms. The protective metabolites may include substances such as lactic acid, acetic acid, ethanol, hydrogen peroxide, bacteriocins, and other small molecular weight metabolites.

GRAS Notification – Use of Lactic Acid Bacteria (continued)
5. Safety and Effectiveness (continued)

These metabolites are normally found in traditional foods cultured with LAB and are considered as GRAS. In addition, some of these substances are listed in GRAS and food standard regulations. Lactic acid is affirmed as GRAS (21 CFR 184.1061 Lactic acid) and allowed in standardized foods such as jams and jellies (21 CFR 150.141 Artificially sweetened fruit jelly and 150.161 Artificially sweetened preserves and jams), cheeses (21 CFR Part 133 Cheeses and related cheese products), and in pickles. Acetic acid is a food (vinegar) and GRAS (21 CFR 182.1 Substances that are generally recognized as safe and 184.1005 Acetic acid) and is allowed in cheeses (21 CFR Part 133 and related cheese products). Hydrogen peroxide is allowed in cheese making (21 CFR 184.1366 Hydrogen peroxide and 133.113 Cheddar cheese) and in processed foods and ingredients (21 CFR 160.105 Dried eggs, 160.145 Dried egg whites, 160.185 Dried egg yolks, 172.814 Hydroxylated lecithin, 172.892 Food starch-modified). In addition, FDA has allowed the use of nisin, an antimicrobial peptide derived from certain strains of *Streptococcus lactis* (reclassified *Lactococcus lactis* subsp. *lactis)* in processed cheese products (21 CFR 184.1538), and from *Lactococcus lactis* subsp. *lactis* as a GRAS substance for ready-to-eat meat and poultry products (GRN 000065; FSIS Directive 7120.1, 12/17/2002).

5.2 Competitive Exclusion

The concept of competitive exclusion for the proposed uses is to use LAB that do not grow at refrigeration temperatures but give an inhibitory effect due to the continuous production of metabolites by the cells during the storage. In general, LAB do not grow at refrigeration temperatures, but if the product is temperature abused, LAB growth and spoilage can serve as an indication of temperature abuse. The LAB strains selected exhibit inhibitory activity toward the target pathogen but do not grow and alter the sensory properties of the food except under temperature abuse conditions. Fresh ground beef was used as the worst-case example. It was important to use ground beef containing the natural flora to determine how the added LAB competes with the natural flora.

Samples were stored at 5° C for 12 days to determine the impact of LAB on the growth and inhibition of *E. coli* O157:H7 (Brashears and Miller 2002). After 4 days of storage, one of the LAB cultures, M35 resulted in significantly lower populations of *E. coli* O157:H7 compared to the control samples. After 8 days of storage, the other 3 LAB cultures resulted in significant differences between control samples and treated samples with more than a 1.5 log difference (> 90% reduction) between the control samples and the treated samples.

GRAS Notification – Use of Lactic Acid Bacteria (continued)
5. Safety and Effectiveness (continued)

They observed a more inhibitory effect against *Salmonella* spp. After 4 days of storage, M35, LA51 and D3 cultures resulted in significant reductions of *Salmonella* compared to the control samples. Each of the cultures resulted in a 1.5 log reduction after 4 days of storage at refrigeration temperatures. After 8 days of storage, M35 resulted in a 2.0 log difference compared to the control and even more inhibition was observed on day 12 with more than a 2.0 log reduction. All LAB cultures except for L7 resulted in significantly lower populations of *Salmonella* compared to the control on days 8 and 12.

Preliminary studies using a combined cocktail containing all 4 cultures at a higher inoculation level suggests that more than a 3.0 log reduction of *E. coli* and a 4.0 log of *Salmonella* can be achieved.

The treatments in the study by Smith et al. (in press) were, 1) ground beef containing both the pathogen and the LAB cultures (NP35, NP 51, NP 3, NP 7), 2) ground beef containing only the pathogen, and 3) a background control containing neither the pathogen or the LAB.

Smith and coworkers conducted a series of studies to determine if four strains of LAB (NP 35, NP 51, NP 3, and NP 7) inhibited *E. coli* O157:H7 and *Salmonella* spp. in ground beef at 5^{o} C and if they had an impact on the sensory properties of the beef. Streptomycin resistant strains were used to facilitate recovery on non-selective media in the presence of the background flora. A 10^7 cfu/ml portion of individual isolates of the LAB were added to tryptic soy broth (TSB) broth containing 10^5 cfu of the pathogen/ml. Samples were stored at 5^{o} C and the numbers of pathogens were determined on days 0, 4, 8 and 12. After 4 days of storage, there were significant reductions in both pathogens exposed to NP 35 and NP 3. After 8 and 12 days of storage, all LAB reduced populations of both pathogens by an average of 3-5 log cycles. A second study was conducted in vacuum-packaged fresh ground beef. The individual LAB isolates resulted in an average reduction of 1.5 log cycles of *E. coli* O157:H7 after 12 days of storage while *Salmonella* was reduced by an average of 3 log cycles. Following this study, a mixed concentrated culture was prepared from all four LAB and added to pathogen inoculated ground beef at a level of 10^8 cfu/g. After 3 days of storage, the mixed culture resulted in a 2.0 log reduction of *E. coli* O157:H7 compared to the control whereas after 5 days of storage, a 3 log reduction occurred. *Salmonella* was reduced to non-detectable levels after day 5. Sensory studies on uninoculated samples indicated that there were no adverse effects on the sensory properties of the ground beef.

GRAS Notification – Use of Lactic Acid Bacteria (continued)
5. Safety and Effectiveness (continued)

5.3 Spoilage

Packaging studies were conducted by Hoyle and coworkers (Hoyle 2005; Hoyle, Brashears and Brooks 2005) comparing traditional packaging and MAP packaging of beef patties at refrigeration (0° C) and abusive temperatures (10° C). The studies were conducted to determine if LAB masked color and odor changes typically associated with the spoilage of ground beef displayed under refrigerated (0° C) or abusive (10° C) temperatures. Microbial and sensory analyses were conducted to determine spoilage endpoints. Packaging consisted of traditional (foam trays overwrapped with permeable film) and MAP packaging (80% O_2 and 20% CO_2). To mimic industry practice, one-half of the MAP samples contained 1000 ppm added rosemary oleoresin. Packages were stored in display cases with a light intensity of approximately 1900 lux.

Samples displayed at 0° C were collected at 0, 24, 48, 72 and 48 hours. Samples displayed at 10° C were collected at 0, 12, 24 and 36 hours. Color tests were conducted on 48 hr and 36 hr samples. Sensory and odor panels were conducted on all sampling intervals. After panel testing, half of the patties were used for microbial analysis and the other half assayed for thiobarbituric acid (TBA) assay. The researchers used six media to isolate and enumerate microorganisms present in the samples. They were Trypticase Soy Agar (nonfastidious and fastidious microorganisms), Pseudomonas F Agar (*Pseudomonas aeruginosa* and other fluorescin-producing pseudomonads), YM Agar (yeasts, molds and other aciduric microorganisms), Violet Red Bile Agar (coliforms), Lactobacilli MRS Agar (*Lactobacillus* spp.), and STAA Agar with supplement SR151E (*Brochothrix thermosphacta*).

GRAS Notification – Use of Lactic Acid Bacteria (continued)
5. Safety and Effectiveness (continued)

At 0° C, traditionally packaged LAB samples had significantly lower yeast and mold (YM) counts than controls throughout display. Among traditional packages, there were no significant differences in coliform, *Brochothrix thermosphatca* (BT), and *Pseudomonas* spp. counts between LAB treatments. At abusive storage temperatures, there were no significant differences in coliform, YM, BT, and *Pseudomonas* spp. counts between traditionally packaged LAB treatments. At 0° C and 10° C, total plate counts and LAB populations in both traditional and MAP packaged inoculated samples were significantly higher than the control. In MAP packaging, no significant differences existed between LAB treatments for YM, coliform, and *Pseudomonas* spp. Samples containing oleoresin had significantly lower coliform and total plate counts at both temperatures. No significant differences in sensory color and odor existed between LAB and controls for traditional and MAP, indicating spoilage was not masked. Furthermore, results indicate rosemary oleoresin inhibits spoilage organism growth in modified atmosphere systems.

Addition of LAB to ground beef at refrigeration temperatures did not significantly ($P > 0.05$) affect the ground beef color in patties stored at 0° C when evaluated by trained panelists (Hoyle 2005). However, the trained panelists did detect a significant ($P < 0.05$) difference over time in patty color. For patties stored at 10° C, trained panelists detected a significant ($P < 0.05$) difference in patty color over time, but no significant ($P > 0.05$) were found between treatments. Although there was a significant difference in color when patties were stored over time, both consumer and trained panelists did not see a significant ($P > 0.05$) difference between uninoculated patties and inoculated patties.

Odor panels were also conducted at sampling times. The trained panelists were asked to identify how strong the smell was and then characterize the smell. Consumer panelists were asked if the meat smelled fresh and would they consume the meat. The trained odor panel determined there was a significant ($P < 0.05$) difference in odor when the patties were stored at 0° C over the sampling period. However, there was not a significant ($P > 0.05$) difference between the uninoculated and inoculated patties stored at 0° C. At 10° C, trained panelists also determined there was a significant ($P < 0.05$) difference in patty odors over time, but did not find a significant difference ($P > 0.05$) between treatments. Consumer panelists responses were significantly ($P < 0.05$) different for patties stored at 0° C over time, but the responses were not significantly ($P > 0.05$) different between treatments. In addition, consumer panelists responses were significantly ($P < 0.05$) different over time for ground beef patties stored at 10° C, but the responses were not significantly ($P > 0.05$) between treatments. As the storage period progressed, consumer responses showed the odor of the meat to not be fresh and those consumers were less likely to eat the patties they had smelled. In addition, as the storage time progressed, trained panelists determined that the strength of the odor had increased.

A study was conducted to determine the effect of ground beef packaged under modified atmosphere conditions consisting of 80% oxygen and 20% carbon dioxide (Hoyle 2005). The ground beef was divided into four different treatment groups which included an uninoculated control, LAB only, LAB with rosemary oleoresin, and an uninoculated control with rosemary oleoresin. A portion of each treatment group was displayed under refrigeration temperatures (0° C) and the remainder was displayed under abusive temperatures (10° C).

For those samples displayed at refrigeration temperatures, samples with added LAB had a significantly higher ($P < 0.05$) total plate count than those without LAB. This is due to the inoculation of the samples with the bacteria. In addition, total plate counts for samples with rosemary oleoresin were significantly lower ($P < 0.05$) than the controls, 4.37 and 4.67, respectively. The LAB samples containing rosemary oleoresin had a significantly higher population of LAB than the other samples, while the control samples without rosemary oleoresin had a significantly lower population of LAB than the remaining samples. There were no significant differences between all treatments in populations of *Pseudomonas* spp. and YM counts. While there was no significant differences in coliform populations for LAB samples, samples containing rosemary oleoresin had a significantly lower coliform count than those samples without the oleoresin.

GRAS Notification – Use of Lactic Acid Bacteria (continued)
5. Safety and Effectiveness (continued)

Trained sensory panelists for samples stored at refrigeration temperatures did not detect differences between samples containing LAB and those that do not. However, the panelists did detect significant differences between samples with or without rosemary oleoresin over the display period. Trained panelists did not detect differences in odor between LAB and control samples, but did detect significant differences in samples containing rosemary oleoresin. No significant differences were found between controls and samples containing LAB by consumer panelists, but they did detect significant differences between samples with rosemary oleoresin and controls throughout the display period. Significant differences in consumer odor panels were found for samples with rosemary oleoresin and controls, but samples with LAB and controls were not different statistically. Hunter color analysis did not detect significant differences for LAB and control samples. L values were not significantly different between samples with rosemary oleoresin and controls. However, rosemary oleoresin and control sample A values were significantly different. Throughout the storage period, B values for samples containing rosemary oleoresin and controls were significantly different.

For those samples stored at abusive temperatures, LAB inoculated samples with and without rosemary oleoresin had significantly higher total plate counts than control samples with and without the oleoresin. The LAB populations throughout the display period were significantly higher in samples with LAB than those without. In addition, significant differences were found between samples with oleoresin and controls throughout the storage period. No significant differences in all treatments were found in *Pseudomonas* spp. Significant differences were found in coliform and YM counts between combinations of LAB and rosemary oleoresin samples and controls.

Trained panelists detected significant differences in color between samples with oleoresin and controls over time, but did not detect differences between LAB samples and controls. No significant differences were found in odor between LAB samples and controls, but trained panelists did detect differences in samples with rosemary oleoresin and controls throughout the storage period. Consumer panelists also found significant differences in color between samples with oleoresin and controls throughout the storage period, but no significant differences were found between LAB samples and controls. Consumer odor panels found significant differences in rosemary oleoresin samples and controls, but did not find differences between LAB samples and controls.

GRAS Notification – Use of Lactic Acid Bacteria (continued)
5. Safety and Effectiveness (continued)

Initial TBA values were not significantly different between treatments. After 24 and 36 hours of display, the treatment groups without added oleoresin had significantly ($P < 0.05$) higher TBA values than those treatment groups with added resin. These results indicate that the addition of rosemary oleoresin slowed lipid oxidation in the presence or absence of LAB.

6. Exposure

In order to estimate the amount of LAB and metabolites are consumed, we have used the product category and RACC values from 21 CFR 101.12 and ¼ lb (113 g) as serving size for ground meat. We have assumed that there are 10^8 cfu of LAB/g of food product, an average consumption of products per week, and that all ground meat and poultry products will contain LAB and metabolites. However, because such ground meat and poultry products will be cooked prior to consumption, there is no added exposure to LAB from these products.

Based on these assumptions, we estimate that there may be no increase in exposure due to use of LAB as a competitive inhibitor in ground meat and poultry products.

Table 1. Estimated Consumption Value for LAB

Food	Serving size, g	LAB/g	Frequency/wk, average	LAB/serving x 10^8
Sausage products	55g	10^8	2	110
Yogurt, etc	225	10^8	2	450
Cheese	30	10^8	2	60
Pickles	30	10^8	1	30
Dietary suppl	1 cap/tab	10^8 to $5x10^8$	2-3/da 14/wk	70
Total				**720**
Ground meats*	113	10^8	3	0

*cooked prior to consumption

GRAS Notification – Use of Lactic Acid Bacteria (continued)

7. Basis for Safety Conclusion

Nutrition Physiology Corporation concludes that the use of LAB mixtures for use as a processing aid in controlling growth of pathogenic microorganisms in raw and processed foods are safe is based on the following:

The history of safe use of LAB species in traditional food and food products using LAB starter cultures.

The use of non-pathogenic LAB strains for the product.

The use of a standard manufacturing process for the LAB using safe and suitable ingredients for culturing and processing the LAB.

The use of LAB as competitive inhibitors in feed for food-producing animals.

The use of LAB as dietary supplements.

Sensory studies indicated that there were no significant differences in color and odor between LAB and controls for traditional and MAP packaging samples.

LAB use did not affect spoilage.

Foods using LAB as competitive inhibitors will be cooked prior to consumption.

GRAS Notification – Use of Lactic Acid Bacteria (continued)

References

Amézquita, A., and M.M. Brashears. 2002. Competitive inhibition of *Listeria monocytogenes* in ready-to-eat meat products by lactic acid bacteria. *J. Food Prot.* 65:316-325.

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Wilderdyke, M.R., D.A. Smith, and M.M. Brashears. 2004. Isolation, identification, and selection of lactic acid bacteria from alfalfa sprouts for competitive inhibition of foodborne pathogens. *J. Food Prot.* 67:947-951.	9

1

Pages 000029 - 000038 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

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4

Competitive Inhibition of *E. coli* O157:H7 and *Salmonella spp.* in Ground Beef Products

Dr. Mindy Brashears
Dr. Mark Miller
Department of Animal and Food Sciences
Texas Tech University

Final Report

Submitted to the Texas Beef Council
and
National Cattleman's Beef Association

October 10, 2002

Competitive Inhibition of *E. coli* O157:H7 and *Salmonella spp.* in Ground Beef Products
-FINAL REPORT-

Principle Investigator
Mindy Brashears, Ph.D.
Mark Miller, Ph.D.

Institution:
Department Animal and Food Sciences
Texas Tech University
Box 42141
Lubbock, TX 79409
(806)742-2469
(806)742-2427 – fax

Project Title:

Competitive Inhibition of *E. coli* O157:H7 and *Salmonella spp.* in Ground Beef Products

Stated Project Objectives:

The overall objective of this study is to eliminate *E. coli O157:H7* and *Salmonella* in ground beef products by competitive inhibition by lactic acid bacteria and thus reduce the number of outbreaks associated with these products.

Background Information about the Need for This Research

According to the Centers for Disease Control (CDC), *E. coli* O157:H7 causes an estimated 73,000 cases of illness each year and 61 deaths. *Salmonella* causes 40,000 reported cases with the estimated actual number of cases being 20 times more than the reported amount. More than 1000 deaths occur each year due to *Salmonella* infection making it the deadliest food-borne pathogen.

Ground beef products are commonly implicated in outbreaks of *Salmonella* and *E. coli* O157:H7. While there are many intervention technologies applied to beef carcasses, very few interventions exist that have been validated to be effective in ground beef products.

Lactic acid bacteria (LAB) are inhibitory towards various pathogenic bacteria and spoilage organisms during growth and refrigerated storage in associative cultures (Brashears et al. 1998; Brashears and Gilliland, 1997; Brashears et al. 1996; Brashears and Durre; Dahiya and Speck, 1967; Gilliland and Speck, 1975; Gilliland and Speck, 1977; Martin and Gilliland, 1980; Price and Lee, 1969; Kao and Frazier, 1966; Shahani et al., 1976). The inhibitory actions were due to the production of acid, hydrogen peroxide, or bacteriocins. Recent reviews of the inhibitory actions of LAB towards food-borne pathogens were done by Barefoot and Nettles (1993), Klaenhammer (1988) and Hopzafel et. al (1995). These reviews suggest that pathogens and spoilage organisms in fermented foods such as yogurt and summer sausage may be inhibited during growth of LAB. Additionally, inhibition can occur during refrigerated storage. Growth of lactic acid bacteria in a fresh meat product would not be desirable, but adding the cells to the meat held at refrigeration temperature could still give an inhibitory effect due to the production of inhibitory substances by the cells. It is possible to select strains of lactic acid bacteria that do not grow at refrigeration temperatures, but produce inhibitory substances. The production of inhibitory compounds by the LAB can continue during storage of the product so there is continuous inhibition of the pathogen during the storage period instead of a one-time reduction that occurs with other antimicrobial interventions (Gilliland and Villegas, 1998; Jaroni and Brashears, 2000).

In a previous study in our laboratory, several strains of lactic acid bacteria were isolated. This strain was selected for its ability to completely eliminate *E. coli* O157;H7, *Salmonella*, and *Listeria monocytogenes* in laboratory media. The unique properties of these organisms is that they eliminate the pathogens at refrigeration temperatures, but it do not grow during refrigerated storage. The organisms are GRAS and can be added to food products.

A comprehensive study was conducted in our laboratory with this LAB to determine if it inhibited *Listeria monocytogenes* in ready-to-eat meat products. All pathogen growth was completely eliminated for the entire 60-day duration of the study in cold cuts and in frankfurters. A second study was conducted with non-pathogen inoculated products to determine if the LAB had a detrimental effect on the sensory properties of the products. For the entire 60-day duration of the sensory study, there were no differences in the control samples and the inoculated samples. Plate counts of LAB in both studies indicated that there was no growth of the LAB during storage, but there was significant inhibition. Further studies have indicated that a protein-based product, most likely a bacteriocin, is produced by this organism at refrigeration temperatures.

Reduction of *E. coli* O157:H7 is an important concern in the beef industry. Ground beef processors currently do not have effective intervention steps in ground beef processes. Intervention steps need to be investigated to ensure the safety of the ground beef supply. This research indicates that the use of competitive inhibition in ground beef products could be an important hurdle to reduce *E. coli* O157 and *Salmonella* in the ground beef supply.

Achievement of the Specific Objectives Stated in the Proposal

All objectives in the original proposal have been achieved.

Materials and Methods

The treatments in this study were, 1) ground beef containing both the pathogen and the LAB, 2) ground beef containing only the pathogen, and 3) a background control containing neither the pathogen or the LAB.

Frozen concentrated cultures of the lactic acid bacteria culture were prepared as described by Brashears et al. (1998) and combined into a "cocktail" mixture and added to the food products. Products were inoculated with the pathogen by adding a cocktail mixture of *Salmonella* spp. or *E. coli* O157:H7 (two separate studies) containing approximately 1×10^4 cfu/ml. Streptomycin resistant (1000ug/mg) strains of the pathogens were used to facilitate recovery of injured cells. The use of these organisms has been validated to be equivalent to non-resistant cells and recovery rates are similar to recovery on non-selective media (Brashears et. al., 2001). Fresh ground beef samples were obtained from the Texas Tech University Meat Laboratory. It is important to use ground beef containing the natural flora to determine if the added LAB competes well in that particular environment. Cells of the LAB were added to the ground beef by pouring a designated amount of a diluted frozen concentrated culture to the products to yield a population of 1×10^7 cfu/g and thoroughly mixing. Background controls received no treatment. All samples were vacuum packaged.

Samples were stored for 10 days at 5°C. Samples were taken on days 4, 8 and 12 and the total number of pathogens present were determined by plating on non-selective media (Trypticase Soy Agar) containing 1000 ug/ml streptymycin on a Spiral Biotech Spiral Plating

System. The numbers were compared to the control that contained only added pathogen, no LAB, to determine the amount of inhibition.

Results and Discussion

Selection of Antibiotic Resistant Organisms

Because we proposed the use of nalidixic acid resistant strains of the pathogens to facilitate recovery of injured cells in nonselective media in the original proposal, we plated uninoculated ground beef on TSA plus 50ug nalidixic acid to ensure that no background flora in the meat would grow on the media. Because a recent project using pork indicated that there were significant background flora in raw pork products that grew in the presence of nalidixic acid, we also tested the beef in media containing streptomycin. We had to identify an antibiotic that suppressed background flora in the raw product.

The raw ground beef samples obtained from several sources were plated on TSA with the following antibiotic concentrations:

a.) TSA plus 50ug nalidixic acid

b.) TSA plus 100 ug nalidixic acid

c.) TSA plus 1000 ug streptomycin

d.) TSA plus 2000 ug streptomycin

The plates were incubated at 37 C for 48 hrs. There was evident growth for all dilutions plated on both the 50 ug and 100 ug nalidixic acid plates. Therefore this antibiotic was not used because it allowed the growth of the background flora.

There was no observed growth for any of the plates containing streptomycin for either the 1000 ug or 2000 ug concentrations. Therefore the lower concentration of streptomycin was used in the study to inhibit background flora in the meat while allowing streptomycin resistant pathogens to grow and recover.

To validate this observation, more ground beef samples were obtained and plated on plates containing streptomycin. The results from this beef sample were the same as the results observed before. There was growth on the 100 ug nalidixic acid plates but there was no growth on either the 1000 ug or 2000 ug streptomycin plates.

In addition to confirming that the background flora would not grow on the media, we also had to be sure that the LAB would not grow and that the pathogens would grow. Four different cultures of *E. coli* O157 and 2 different lactic acid bacteria cultures on pre-poured TSA plates. One set of plates contained 1000 ug streptomycin and the other set contained 2000 ug of streptomycin.

After 48 hrs. of incubation at 37C all of the *E. coli* cultures grew confirming their antibiotic resistance. Neither of the lactic acid bacteria cultures grew. Therefore the media containing 1000 ug of streptomycin was used to suppress the background flora in the meats.

<u>Inhibition in Ground Beef</u>

We stored samples at 5 C for 12 days to determine the impact of Lactic Acid Bacteria (LAB) on the growth and inhibition of *E. coli* O157:H7. After 4 days of storage, one of the LAB cultures, M35 resulted in significantly lower populations of *E. coli* O157:H7 compared to the control samples (Figure 1). After 8 days of storage, the other 3 LAB cultures resulted in significant differences between control samples and treated samples with more than a 1.5 log difference (>90% reduction) between the control samples and the treated samples.

A more inhibitory effect was observed against *Salmonella* spp. After 4 days of storage, M35, LA51 and D3 resulted in significant reductions of Salmonella compared to the control samples (Figure 2). Each of these resulted in a 1.5 log reduction after 4 days of storage at refrigeration temperatures. After 8 days of storage, M35 resulted in a 3.5 log difference compared to the control and even more inhibition was observed on day 12 with more than a 4 log reduction. All LAB cultures except for L7 resulted in significantly lower populations of *Salmonella* compared to the control on days 8 and 12.

While we did observe significant reductions in this process, a higher amount of reduction is desirable. Currently we are continuing with this study to examine the behavior of *E. coli* and *Salmonella* in the presence of a combined cocktail containing all 4 cultures at a higher inoculation level. Preliminary evidence indicates that we can achieve more than a 3 log reduction of *E. coli* and a 4 log of *Salmonella* using this approach.

Additionally, we plan to study the effects on the sensory properties of the product in non-inoculated samples to determine if the use of the LAB is feasible from a quality perspective.

Publications

- Manuscript will be submitted before the end of the year to Journal of Food Protection.
- Presentation/abstract will be presented at the 2003 meeting of the International Association of Food Protection

Lay Interpretation

Lactic acid bacteria (LAB), similar to cultures used to produce cheese and yogurt, were added to ground beef to determine if the cultures inhibited *E. coli* O157:H7 and *Salmonella* during refrigerated storage. Some of the cultures reduced *E. coli* O157:H7 by more than 90% and *Salmonella* by more than 99.9%. Adding the cultures to ground beef may be an effective strategy to control food-borne pathogens in ground beef product.

Figure 1. Competitive inhibition of *E. coli* O157 at 5 C in during a 12 day storage period

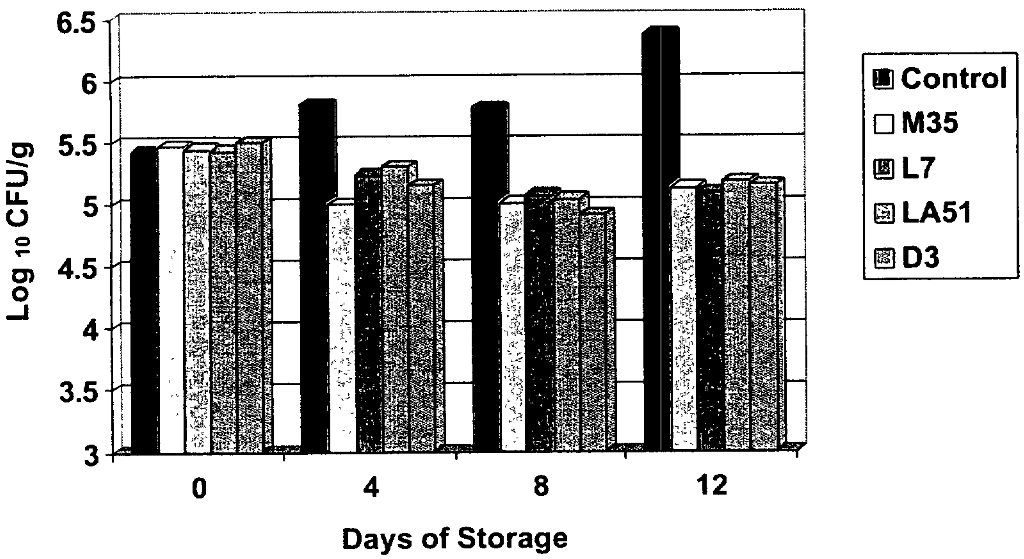

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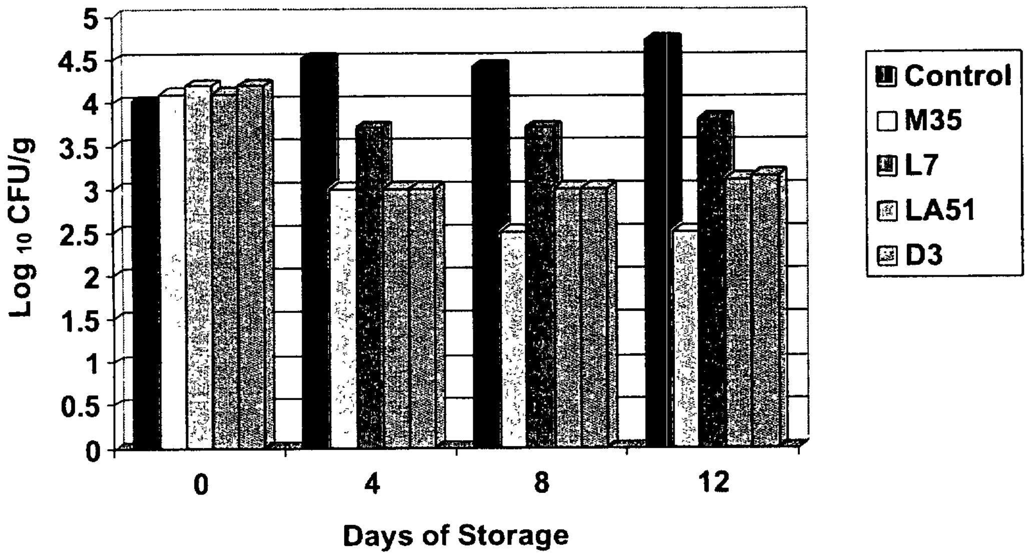
Figure 2. Competitive inhibition of *Salmonella* spp. at 5 C in ground beef during a 12 day storage period



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Tab 5

Hoyle, A.R. 2005. Spoilage characteristics of ground beef with added lactic acid bacteria at abusive and refrigerated temperatures packaged in modified atmosphere and traditional packaging. Master's Thesis, Texas Tech University.

See:

http://etd.lib.ttu.edu/theses/available/etd-05032005-151138/unrestricted/amyhoyle.pdf

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Submission End

000163

AM

Garcia, Edmundo

From: CLYDE TAKEGUCHI [ctakeguchi@phoenixrising.com]
Sent: Wednesday, November 09, 2005 3:56 PM
To: Garcia, Edmundo
Cc: mindy.brashears@ttu.edu; dware@bovamine.com
Subject: RE: Nutrition Physiology GRN on Lactic Acid Bacteria

Dear Edmundo:

We apologize for not including the citation in the list of references. The article citation is: Wolf, B. W., Garleb, K. A., Ataya, D. G. & Casas, I. A. (1995). Safety and tolerance of Lactobacillus reuteri in healthy adult male subjects. Microbial Ecology in Health and Disease 8, 41-50.[ISI]

Let me know if you have any additional questions.

Regards,

Clyde Takeguchi
Phoenix Regulatory Associates, Ltd.

-----Original Message-----
From: Garcia, Edmundo [mailto:Edmundo.Garcia@cfsan.fda.gov]
Sent: Wednesday, November 09, 2005 2:00 PM
To: CLYDE TAKEGUCHI
Subject: RE: Nutrition Physiology GRN on Lactic Acid Bacteria

Dr. Takeguchi,

In the information that you submitted in response to our questions, we noticed that, in page 2 section 2, you cite a study by Wolf et. al. The information referencing this study is not listed in the "References" section of your document. Could you please provide the complete reference for this study?

We are in the process of completing our review and your prompt response would be greatly appreciated.

Thanks,

Edmundo Garcia Jr.
Consumer Safety Officer
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition U.S. Food & Drug Administration 301-436-1189

-----Original Message-----
From: CLYDE TAKEGUCHI [mailto:ctakeguchi@phoenixrising.com]
Sent: Thursday, September 01, 2005 3:27 PM
To: Garcia, Edmundo
Cc: Jeff.Canavan@fsis.usda.gov; dware@bovamine.com; mindy.brashears@ttu.edu
Subject: RE: Nutrition Physiology GRN on Lactic Acid Bacteria

Dear Edmundo:

000168

This is to confirm that the GRN was submitted on the basis of scientific procedures because this is a new use of an old process.

The use of LAB as starter cultures in fermented food products has a long history of safe use. Although the technical effect of LAB has been known for a long time, the use of LAB as protective cultures in food products has become more common recently. We have provided you with a with a more substantial safety statement supporting Nutrition Physiology Corporation's (NPC) conclusion that the use of LAB as protective cultures in food products is considered generally recognized as safe (attached). The reference list includes all articles reviewed but not all the articles are referenced in the safety statement. Please let us know if you need a copy of any of the references.

NPC agrees with USDA's conclusion that ingredient labeling should be required. NPC will be contacting USDA in the near future to discuss issues on suitability studies in poultry, labeling, and test marketing of LAB-containing products.

If you have any additional questions, you can call me at (703) 406-0906, or Dr. Douglas Ware at (800) 993-9899.

Regards,

Clyde A. Takeguchi, Ph.D.
Executive Vice President
Phoenix Regulatory Associates, Ltd.
21525 Ridgetop Circle
Suite 240
Sterling, VA 20166
Phone: 1-703-406-0906
Fax: 1-703-406-9513

(b)(5)

[redacted]

(b)(5)

-----Original Message-----
From: CLYDE TAKEGUCHI [mailto:ctakeguchi@phoenixrising.com]
Sent: Tuesday, July 26, 2005 3:24 PM
To: Edmundo.Garcia@cfsan.fda.gov
Cc: Ruxanne Baines
Subject: Nutrition Physiology GRN on Lactic Acid Bacteria

Dear Edmundo:

I wanted to check on the status of the GRN submitted by Nutrition Physiology.

They have a customer that is interested in doing a trial with their LAB product. I was wondering whether such a trial can be done prior to a GRN determination.

Regards,

Clyde Takeguchi
Phoenix Regulatory Associates, Ltd.
(703) 406-0906

Garcia, Edmundo

From: CLYDE TAKEGUCHI [ctakeguchi@phoenixrising.com]
Sent: Tuesday, November 29, 2005 1:37 PM
To: Garcia, Edmundo
Cc: Orstan, Aydin; Dinovi, Michael J; dware@bovamine.com
Subject: RE: Response to Additional Questions

Edmundo:

As you know, they have used the LAB mixture on experimental samples. In experimental trials conducted by Dr. Brashears' lab, the LAB concentrate was diluted with water and poured or sprayed on the ground meat and mixed. For the final commercial product, the lyophilized product would be reconstituted in water and sprayed or poured on the ground beef and mixed.

I hope this helps.

Regards,

Clyde

-----Original Message-----
From: Garcia, Edmundo [mailto:Edmundo.Garcia@cfsan.fda.gov]
Sent: Saturday, November 26, 2005 1:36 PM
To: CLYDE TAKEGUCHI
Cc: Orstan, Aydin; Dinovi, Michael J; dware@bovamine.com
Subject: RE: Response to Additional Questions

Clyde,

How is the LAB mixture added to the final product?

Edmundo Garcia Jr.
Consumer Safety Officer
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition U.S. Food & Drug Administration

-----Original Message-----
From: CLYDE TAKEGUCHI [mailto:ctakeguchi@phoenixrising.com]
Sent: Tuesday, November 22, 2005 11:52 AM
To: Edmundo.Garcia@cfsan.fda.gov
Cc: aydin.orstan@fda.hhs.gov; michael.dinovi@fda.hhs.gov; dware@bovamine.com
Subject: Response to Additional Questions

Dear Edmundo, Aydin, and Mike:

Here are the answers to the questions on specifications for the LAB, carrier used in the final product, and the level of use of LAB.

1. Non lactic acid bacteria tolerance is < 500 cfu per gram of finished product. 2. Tolerances for Staph aureus and Salmonella are negative 3. Carrier is food grade lactose 4. Recommended dosage is 10>7 total cfu per gram of fresh meat. Range is 10>6 to 10>8.
5. 500 gram package will contain 10>13 cfu per gram to treat one ton (2000 pounds) of fresh meat.

Let me know if you have any additional questions.

Regards,

Clyde

000171

Supplement to GRN 000171

GRAS Notification for the Use of
Lactic Acid Bacteria to Control Pathogenic Bacteria
in Meat and Poultry Products

Volume 1 of 1

Submitted by:

Nutrition Physiology Corporation
11358 Woods Bay Lane
Indianapolis, IN 46236

July 20, 2006

[redacted]

[redacted]

Cover Letter

[redacted]

PHOENIX
REGULATORY ASSOCIATES, LTD.
Washington DC Headquarters
21525 Ridgetop Circle
Suite 240
Sterling, Virginia 20166

European Office: Dorset, England

July 20, 2006

Edmundo Garcia, Jr., Consumer Safety Officer
Office of Food Additive Safety (HFS 255)
Center for Food Safety and Applied Nutrition
5100 Paint Branch Parkway
College Park, MD 20740

VIA: Federal Express

Re: GRAS Notice No. GRN 000171: Supplement

Dear Mr. Garcia:

On December 7, 2005, FDA issued a letter to Nutrition Physiology Corporation (NPC) stating that it had no questions regarding NPC's conclusion that the Lactic Acid Bacteria (LAB) Mixture is GRAS for control of pathogenic bacteria in fresh chopped/ground, whole muscle cuts, and carcasses of meat and poultry at use levels between 10^6 to 10^8 colony forming units of lactobacilli per gram of product.

On May 4, 2006, NPC requested that FSIS conduct an acceptability determination for the use of LAB in ready-to-eat (RTE) meat products. As part of their evaluation on the expanded use of GRAS substances, they contacted you regarding FDA's safety assessment. You requested an exposure assessment for the added use of LAB in RTE products to supplement GRN 000171.

We have attached our rationale for determining that there will be minimal change in exposure to LAB when used in RTE products. Supporting data was published in a 2002 Journal of Food Protection article titled, "Competitive Inhibition of *Listeria monocytogenes* in Ready-to-Eat Meat Products by Lactic Acid Bacteria," by A. Amézquita and M. Brashears. The article was submitted as Reference 1 in the original GRN.

If you have any questions or require additional information, please contact me by telephone at (703)-406-0906 or by email at phoenix@phoenixrising.com.

Sincerely,

[redacted] (b)(6)

Clyde A. Takeguchi, Ph.D.
Executive Vice President

Enclosure: Estimated Consumption of LAB when used in RTE Products (two copies)

cc: R. Post, FSIS (two copies)
D. R. Ware, Ph. D., Nutrition Physiology Corporation
M. M. Brashears, Texas Tech University

Phone 703-406-0906 • Facsimile 703-406-9513
http://www.phoenixrising.com • email: phoenix@phoenixrising.com

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Attachment

Amézquita, A., and Brashears, M. M., "Competitive Inhibition of *Listeria monocytogenes* in Ready-to-Eat Meat Products by Lactic Acid Bacteria," J. Food Prot., 65:316-325 (2002)	000009

Estimated Consumption

Estimated Consumption of LAB when Used in Ready-to-Eat (RTE) Products

Table 1. Estimated Consumption Value for LAB

Food	Serving size, g	LAB/g	Frequency/wk, average	LAB/serving x 10^8
Sausage products	55g	10^8	2	110
Yogurt, etc	225	10^8	2	450
Cheese	30	10^8	2	60
Pickles	30	10^8	1	30
Dietary supplements	1 cap/tab	10^8 to $5x10^8$	2-3/da 14/wk	70
Total				**720**
Ground meats*	113	10^8	3	0
RTE products	55	10^8	2	110

*cooked prior to consumption

<u>Assumptions</u>

RTE products are in the same category as the sausage products.

Amount added, 10^7 LAB/g to the surface of all products (by dipping or spraying).

LAB do not grow at refrigeration temperatures but continue to produce metabolites during refrigerated storage.

The LAB population will be about the same when consuming RTE products with or without LAB treatment, because there will be minimal change in LAB exposure with RTE products. (Amézquita, A., and M.M. Brashears. 2002, attached)

<u>Conclusion</u>

There will be no increase in the exposure to LAB by consumers due to use of LAB as a competitive inhibitor to control pathogenic microorganisms in RTE products.

Attachment

Pages 000191 - 000200 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Reference List for Industry Submission, GRN 000171

Pages	Author	Title	Publish Date	Publisher	BIB_Info
000029 - 000038	Amezquita, A. ; Brashears, M.M.	Competitive Inhibiton of Listeria monocytogenes in Ready-to-Eat Meat Products by Lactic Acid Bacteria	2002	Journal of Food Protection	Volume 65, Number 2, pgs 316 - 325
000040 - 000095	Brashears, Mindy M.; Amezuita, Alejandro; Jaroni, Divya	Lactic Acid Bacteria and Their Uses in Animal Feeding to Improve Food Safety	NA	NA	NA
000097 - 000105	Brashears, M.M.; Jaroni, D.; Trimble, J.	Isolation, Selection, and Characterization of Lactic Acid Bacteria for a Competitive Exclusion Product To Reduce Shedding of Escherichia coli O157:H7 in Cattle	2003	Journal of Food Protection	Volume 66, Number 3, pgs 355 - 363
000121	Hoyle, Amy R.; Brashears, Mindy M.; Brooks, J. Chance	The Effect of Lactic Acid Bacteria, Rosemary Oleoresin, and Package Type on the Spoilage of Temperature Abused Ground Beef.	NA	NA	NA
000123 - 000127	Reilly, S.S.; Gilliland, S.E.	Inhibition of Escherichia Coli O157:H7 By Lactobacillus Acidophilus Isolated From Calves	NA	NA	NA
000129 - 000155	Smith, L; Mann, J.E.; Harris, K.; Miller,M.F.; Brashears, M.M.	Reduction of E. coli O157:H7 and Salmonella spp. in Ground Beef using Lactic Acid Bacteria and the Impact on Sensory Properties	NA	NA	pgs 2-27
000158 - 000162	Wilderdyke, M.R.; Smith, D.A.; Brashears, M.M.	Isolation, Identification, and Selection of Lactic Acid Bacteria from Alfalfa Sprouts for Competitive Inhibition of Foodborne Pathogens	2004	Journal of Food Protection	Volume 67, Number 5, pgs 947-951
000191 - 000200	Amezquita, A.; Brashears, M. M.	Competitive Inhibition of Listeria monocytogenes in Ready-to-Eat Meat Products by Lactic Acid Bacteria	2002	Journal of Food Protection	Volume 65, Number 2, pgs 316-325

NA- Not applicable